

## TROPHIC CHANGES IN THE SKELETAL MUSCLES OF RATS AFTER THERAPY WITH SILDENAFIL AND CEREBROLYSIN IN THE LOWER LIMB ISCHEMIA MODEL

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For many patients with lower limb ischemia, surgical treatment is not beneficial. We have studied the efficacy of combination therapy with sildenafil (Viagra) by Pfizer, France, and cerebrolysin (Ever Neuro Pharma, Austria) for lower limb ischemia in the Wistar rat model. The animals were divided into 6 groups (20 rats each): intact animals; sham-operated animals; rats with ischemia and no treatment administered (controls); rats with ischemia who received a 28-day monotherapy with sildenafil (2.2 mg/kg orally); rats with ischemia who received a 28-day monotherapy with 0.005 ml cerebrolysin; rats with ischemia who received a combination therapy with 2.2 mg/kg sildenafil for 7 days and 0.005 ml i. m. cerebrolysin for 10 days. Microcirculation in shin muscles was evaluated on days 21 and 28 of the experiment. On the same days, rats were overdosed with anesthetics and sacrificed in tens. Then, histological sections of shin muscles were prepared. Regional blood flow was significantly higher ( $p < 0.05$ ) in three experimental groups, compared to the controls; however, the combination therapy was far more effective than monotherapy, regardless of the medication used. Macroscopically, the muscles of the animals included into the experimental groups did not differ from the muscles of the intact animals; microscopically, no necrotic lesions were observed in the experimental groups that were typical for the ischemized rats who had received no treatment. Neovascularization was also observed in the experimental groups.

**Keywords:** lower limb ischemia, ischemia treatment, sildenafil, cerebrolysin, pharmacotherapy, combination therapy

**Acknowledgements:** authors thank professor Victor Lazarenko (Kursk State Medical University) and professor Alexandr Khudin (Kursk State University) for providing research facilities for the experiment.

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**Received:** 20.08.2016 **Accepted:** 27.08.2016

## ТРОФИЧЕСКИЕ ИЗМЕНЕНИЯ СКЕЛЕТНОЙ МУСКУЛАТУРЫ КРЫС ПОСЛЕ ФАРМАКОТЕРАПИИ СИЛДЕНАФИЛОМ И ЦЕРЕБРОЛИЗИНОМ ПРИ МОДЕЛИРОВАНИИ ИШЕМИИ НИЖНИХ КОНЕЧНОСТЕЙ

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Ишемия нижних конечностей — заболевание, в большом числе случаев не поддающееся хирургическому лечению. Нами была исследована эффективность комбинированной фармакотерапии силденафилом (Viagra, Pfizer, Франция) и «Церебролизином» (EVER Neuro Pharma, Австрия). Ишемию мышц голени моделировали на крысах линии Wistar. Сформировали 6 групп животных по 20 особей: интактные; ложнооперированные; с ишемией и без лечения (контрольная группа); с ишемией и монотерапией силденафилом (перорально 2,2 мг/кг в течение 28 дней); с ишемией и монотерапией «Церебролизином» (внутримышечно 0,005 мл в течение 20 дней); с ишемией и комбинированной терапией (силденафил — перорально 2,2 мг/кг в течение 7 дней, «Церебролизин» — внутримышечно 0,005 мл в течение 10 дней). Измеряли уровень микроциркуляции крови в мышцах голени на 21-е и 28-е сутки. В эти же сроки выводили из эксперимента путем передозировки наркоза по 10 животных и готовили гистологические препараты мышц голени. Уровень регионарного кровотока достоверно ( $p < 0,05$ ) повышался в трех опытных группах по сравнению с контрольной, однако при этом комбинированная терапия была значительно эффективнее монотерапии независимо от препарата. Макроскопически мышцы животных опытных групп не отличались от мышц интактных животных, микроскопически — наблюдалось отсутствие некротических участков, характерных для ишемизированных мышц крыс, не получавших лечения, а также новообразование сосудов.

**Ключевые слова:** ишемия нижних конечностей, коррекция ишемии, силденафил, церебролизин, фармакотерапия, комбинированная терапия, ФДЭ-5

**Благодарности:** профессору Виктору Лазаренко из Курского государственного медицинского университета и профессору Александру Худину из Курского государственного университета за возможность выполнения эксперимента на базе научно-исследовательских лабораторий их университетов.

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**Статья поступила:** 20.08.2016 **Статья принята к печати:** 27.08.2016

Lower limb ischemia is a chronic arterial occlusion occurring in the legs caused by atherosclerosis, obliterating endarteritis and diabetes [1, 2]. Operative techniques used for its treatment include surgery through skin incisions (shunting and endarterectomy) and various minimally invasive interventions (X-ray-controlled angioplasty and stenting) that restore arterial patency in case the artery is totally blocked or improve blood flow if the blockage is incomplete. However, surgery can be recommended for half of patients only [3–6].

A solution to this problem is drug therapy. The most effective medication for treating critical limb ischemia is Vasaprostan by UCB Pharma, Germany; its active ingredient is alprostadil, a synthetic analogue of natural prostaglandin E1. However, it does not work as a vasodilator that typically widens blood vessels and improves peripheral circulation; Vasaprostan induces changes in blood biochemistry when circulating in blood for a long time [7].

There are a number of medications that affect lipid exchange, peripheral vascular beds and rheological properties of blood, but they do not eliminate vasospasm, a key factor in the progression of critical limb ischemia. Hopes are raised by a new class of drugs that facilitate vasodilation using the effect of endogenous nitric oxide (NO); the latter is produced by nerve endings and endothelial cells and intensifies synthesis of intracellular alarmone, namely, cyclic guanosine monophosphate (cGMP). The same effect can be achieved by using sildenafil, a phosphodiesterase type 5 inhibitor (PDE5) and a cGMP-hydrolyzing enzyme [1, 2, 8–10].

There has been a growing interest in Cerebrolysin (EVER Neuro Pharma, Austria), a drug used to treat stroke, Alzheimer disease and traumas to the brain. It was shown to reduce enzymatic activity of superoxide dismutase and catalase, which are two basic enzymes activated through oxidative stress. However, Cerebrolysin works indirectly by reducing production of superoxide anion and hydrogen peroxide that are substrates for the above mentioned enzymes. Besides, Cerebrolysin was shown to inhibit formation of hydroxyl radicals [11–13]. Also, studies *in vitro* and *in vivo* demonstrated that the drug inhibits calpain activity by 60 %, which means that fewer cells undergo apoptosis [14].

The aim of this work was to assess efficacy of lower limb ischemia therapy with sildenafil (Viagra, Pfizer, France) and Cerebrolysin in a mouse model.

## METHODS

The experiment was carried out in pre-quarantined Wistar rats (age of 4 months, weight of 230–260 g) provided by the Research Institute of Ecological Medicine of Kursk State Medical University. For this study, healthy animals were selected. They were kept in a standard experimental biological cleanroom at 22–24 °C under 12 : 12 cyclic lighting conditions. All rats received food pellets and filtered tap water. Procedures were performed at a fixed time in the afternoon. Anesthesia was performed intra-abdominally using 300 mg/kg of chloral hydrate aqueous solution; animals were sacrificed by its overdose. The experiment was carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986).

To allocate animals to different groups, stratified randomization was used. Stratification factors were body weight and procedures performed on the animals. The following groups were formed:

- 1) intact animals (n = 20),
- 2) sham-operated animals (n = 20),

3) animals with crural muscle ischemia who received no treatment (the control group, n = 20),

4) animals with crural muscle ischemia who received sildenafil (n = 20),

5) animals with crural muscle ischemia who received Cerebrolysin (n = 20),

6) animals with crural muscle ischemia who received sildenafil and Cerebrolysin (n = 20).

The group of sham-operated animals was formed of rats that had been incised lengthwise on the inner thigh under anesthesia, their neurovascular bundle was isolated and the incision was then closed by continuous sutures.

Ischemia of crural muscles was modeled in anesthetized animals in the supine position. The hair on the inner thigh was accurately removed; the skin was washed with 70 % surgical spirit. The incision was done on the inner thigh lengthwise. Elements of the thigh neurovascular bundle were isolated. The artery was separated from the vein and the nerve, a ligature was applied to its upper section where *arteria saphena*, an analogue of deep femoral artery in humans, branches off (under inguinal ligament) [15]. *A. saphena* was ligated and transected. The popliteal artery and upper crural arteries (anterior and posterior tibial arteries) were isolated and transected without ligation. Then, femoral artery was transected 3 mm below the previously applied ligature. The section of the magistral vessel including the femoral and popliteal arteries and upper anterior and posterior crural arteries was removed. No retrograde bleeding from crural arteries was observed. The wound was closed by a continuous suture [16].

The animals from groups 4 and 6 were administered 2.2 mg/kg sildenafil citrate per os for 28 and 7 days, respectively. The animals from groups 5 and 6 received i. m. injections of 0.005 ml Cerebrolysin for 20 and 10 days, respectively [17]. Blood microcirculation in crural muscles was assessed on days 21 and 28 of the experiment using MP100 data acquisition system in LDF 100C laser Doppler flowmetry mode (LDF) and TSD144 needle probe for invasive measurements (all by Biopac Systems, USA). LDF data were recorded and processed using Acqknowledge 3.8.1 software (Biopac Systems); microcirculation was measured in perfusion units (PU). To build a microcirculation curve, data from five different regions of the muscle were recorded for 30 s at each point: the middle point of the muscle longitudinal axis, two points 3–5 mm above it and below, a more lateral and a more medial point with regard to the first point.

The animals were sacrificed in tens by anesthetic overdose on days 21 and 28 of the experiment. In each case, autopsy was performed and crural muscle slices were prepared. Samples for histological analysis were fixed in 10 % formalin for 7 days. Paraffin blocks and microsections were prepared using a standard technique. Slices were stained with hematoxylin, eosin and Van Gieson's stain, and then studied with the Levenhuk 320 microscope (Levenhuk, USA). For morphological analysis, sections were imaged using Levenhuk C310 digital camera and ScopeTek ScopePhoto 3.1.268 software (Hangzhou Scopetek Opto-Electronic Co., China). Using 4 × 20 × 6 magnification, quantitative changes in the vessels were studied within the field of view.

Considering reports on compensatory restoration of regional blood flow in the model selected for this study, we concluded that the most comprehensive data were obtained on day 28 of the experiment. Data obtained on day 21 were considered interim [18].

Statistical processing was done using Microsoft Excel 10.0. Mean values (M) and standard error of mean (m) were



calculated. To compare measurement values obtained from different groups and to determine if differences between them were significant, we used a two-sample t-test with variances. Differences were considered significant with  $p < 0.05$ .

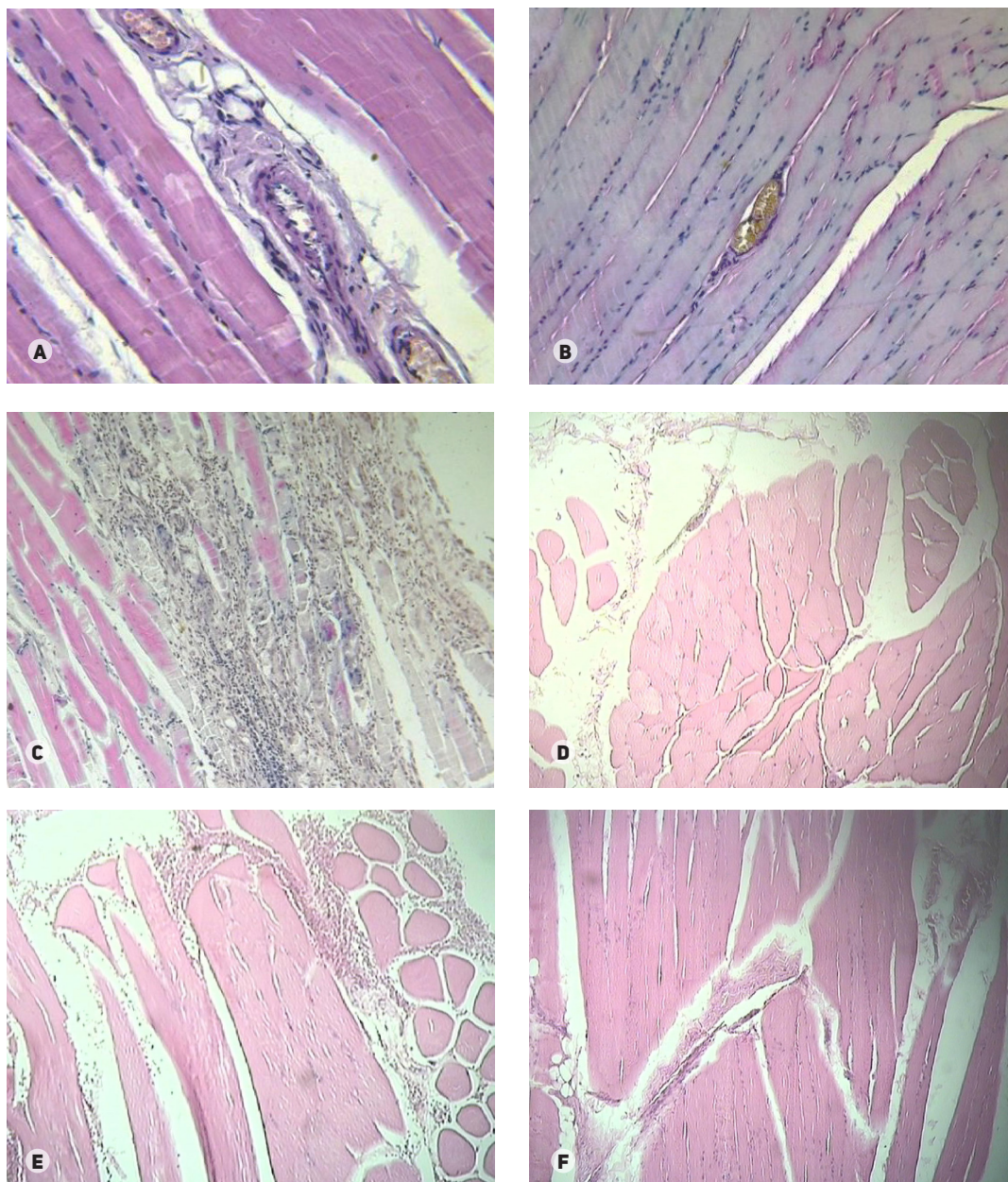
## RESULTS

The mean perfusion value in the crural muscles of the intact animals on day 21 of the experiment was  $527 \pm 13$  PU. Histological analysis revealed densely packed bundles of monocytes, with plethoric venules and arterioles inside containing very few erythrocytes. Lumens were wide; no degrading changes in endothelial cells were observed (fig. A).

In the group of sham-operated animals, mean perfusion value did not differ significantly from that of the intact group, and was  $519 \pm 13$  PU on day 21 of the experiment ( $p = 0.66$ ) and  $521 \pm 16$  PU on day 28 of the experiment ( $p = 0.77$ ). No difference in tissue morphology was detected (fig. B).

In the group of animals with untreated crural muscle ischemia, mean perfusion values were significantly lower, compared to the group of intact animals:  $325 \pm 3$  PU on day 21 of the experiment ( $p < 0.05$ ) and  $371 \pm 2$  PU on day 28 of the experiment ( $p < 0.05$ ). On day 21 damaged muscles were swollen, with large grey and brown patches. Histological analysis showed that those were necrotic foci with resorbed necrotic fibers and proliferating granulation tissue. On day 28, the color went back to normal, but the muscles still looked hypotrophic. Areas of resorbed necrotic tissue were considerably smaller. Formation of individual capillaries and vascular plethora were observed in the microcirculatory bed. There were patches of atrophied muscle fibers close to the necrotic foci (fig. C).

Sildenafil contributed to a statistically significant increase in regional blood flow to the ischemized crural muscles, compared to group 3: perfusion was  $425 \pm 4$  PU on day 21 ( $p < 0.05$ ) and  $803 \pm 10$  PU on day 28 ( $p < 0.05$ ). Perfusion values in group 4 on day 21 were close to those in the group



Morphological analysis of rat crural muscles. (A) Intact rats. (B) Sham-operated rats (group 2). (C) Controls. (D) Rats treated with sildenafil (group 4). (E) Rats treated with Cerebrolysin (group 5). (F) Rats treated with sildenafil and Cerebrolysin (group 6). Staining: hematoxylin and eosin,  $\times 140$

of intact animals; on day 28 they were considerably higher. Macroscopically, ischemized muscles did not differ in color or appearance from the muscles of the intact rats. No necrotic changes were detected by microscopy, but we observed rare patches of atrophic myocytes and cell proliferation (fig. D).

In the group of animals with crural muscle ischemia treated with Cerebrolysin, a statistically significant increase in perfusion was observed, compared to group 3:  $429 \pm 12$  PU on day 21 ( $p < 0.05$ ) and  $767 \pm 8$  PU on day 28 ( $p < 0.05$ ). On day 21, we noticed grey and brown patches — large necrotic foci with resorbed necrotic fibers and proliferating granulation tissue. On day 28 ischemized muscles did not differ in color or appearance from the muscles of the intact rats. Microscopy did not detect any necrotic changes in the. Formation of individual capillaries and vascular plethora were observed in the microcirculatory bed (fig. E).

In rats treated with a combination of sildenafil and Cerebrolysin, mean perfusion value was  $754 \pm 9$  PU on day 21 ( $p < 0.05$  compared to the control group); on day 28 it went up to  $1004 \pm 13$  PU ( $p < 0.05$ ). Morphologically, the muscles here did not differ from those treated with sildenafil (fig. F).

## DISCUSSION

The obtained results indicate that both treatment types — a combination therapy of sildenafil and Cerebrolysin and monotherapy - significantly stimulate regional blood flow in rats with crural muscle ischemia. However, a combination therapy is more effective in stimulating formation of collateral blood vessels.

Sildenafil is a PDE5 inhibitor, an enzyme involved in different biochemical processes inside the cell. Over the past decades it has been discovered that PDE5 inhibitors can be used for treating various pathologies [19]. Sildenafil triggers a cascade that activates protein kinase C and elevates intracellular levels of cGMP in cardiomyocytes due to the activation of inducible and endothelial nitric oxide synthases. A resulting cardioprotective

effect is mediated by the opening of mitochondrial ATP-sensitive potassium channels (mitoKATP-channels) [20]. The opening of mitoKATP-channels increases membrane potential of the myocardial cells and accelerates ATP synthesis and  $Ca^{2+}$  transport across the membrane. Smooth muscle cells relax, arterial lumens widen and blood flow increases [21].

It is the mitoKATP-channels that play a key role in the mechanism of anti-ischemic protection. They are found in many organs, including the vasculature. Their activity was first observed in sarcolemma (sarcoKATP-channels) and then in mitochondria. In both cases, their activity is affected by physiological concentrations of ATP. The channels open when ATP concentration goes down significantly or the adenine nucleotide content decreases. Thus, the channels are a sensor of oxygen and glucose (ATP sources) supply. We think that cytoprotective effect of sildenafil can be explained by the activity of KATP-channels [21]. Perhaps, sildenafil also stimulates neoangiogenesis in the ischemized rat muscle. The evidence here is the results of LDF and the morphological analysis: on day 28 compensatory restoration occurred followed by the increase in regional blood flow to the ischemized muscles of the experimental animals caused by angiogenesis. Earlier we mentioned that Cerebrolysin inhibits enzymic activity of superoxide dismutase and catalase (antioxidative effect) and exhibits anti-apoptotic properties. Besides, the drug has an anti-inflammatory effect [22, 23]. Thus, Cerebrolysin facilitates sildenafil-induced stimulation of natural mechanisms of angiogenesis.

## CONCLUSIONS

We have demonstrated high efficacy of a combination therapy with sildenafil and Cerebrolysin in rats with lower limb ischemia. Measured in perfusion units, perfusion in the ischemized muscles of experimental rats on day 28 was almost twice as high as in the control group.

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